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Sonic hedgehog is an endodermal signal inducing *Bmp-4* and *Hox* genes during induction and regionalization of the chick hindgut

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SUMMARY

Reciprocal inductive signals between the endoderm and mesoderm are critical to vertebrate gut development. *Sonic hedgehog* encodes a secreted protein known to act as an inductive signal in several regions of the developing embryo. In this report, we provide evidence to support the role of *Sonic hedgehog* and its target genes *Bmp-4* and the *Abd-B*-related *Hox* genes in the induction and patterning the chick hindgut.

***Sonic* is expressed in the definitive endoderm at the earliest stage of chick gut formation. Immediately subjacent to *Sonic* expression in the caudal endoderm is undifferentiated mesoderm, later to become the visceral mesoderm of the hindgut. Genes expressed within this tissue include *Bmp-4* (a *TGF-β* relative implicated in proper growth of visceral mesoderm) and members of the *Abd-B* class of *Hox* genes (known regulators of pattern in many aspects of development). Using virally mediated misexpression, we show that Sonic hedgehog is sufficient to induce ectopic expression of *Bmp-4* and specific *Hoxd* genes within the mesoderm. Sonic therefore appears to act as a signal in an epithelial-mesenchymal interaction in the earliest stages of chick hindgut formation.**

Gut pattern is evidenced later in gut morphogenesis with the presence of anatomic boundaries reflecting phenotypically and physiologically distinct regions. The expression pattern of the *Abd-b*-like *Hox* genes remains restricted in the hindgut and these *Hox* expression domains reflect gut morphologic boundaries. This finding strongly supports a role for these genes in determining the adult gut phenotype.

Our results provide the basis for a model to describe molecular controls of early vertebrate hindgut development and patterning. Expression of homologous genes in *Drosophila* suggest that aspects of gut morphogenesis may be regulated by similar inductive networks in the two organisms.

Key words: gut, hindgut, induction, Hox, *Sonic hedgehog*, *Shh*, *Bmp-4*

INTRODUCTION

The gastrointestinal tract performs essential functions required for nutrition and hydration, and provides the anlage for specialized organs needed for respiration and hormone production. The gut is formed by similar morphogenic processes in all vertebrates. In the chick, gut morphogenesis begins at stage 8 (Hamburger and Hamilton, 1951) when differential growth produces a ventral in-folding of the anterior definitive endoderm to form the anterior intestinal portal (AIP) (Romanoff, 1960). The AIP lengthens posteriorly forming the foregut. A second wave of endodermal invagination is initiated posteriorly at stage 13, creating the caudal intestinal portal (CIP). The CIP extends anteriorly forming the hindgut. Ultimately these tubes meet and fuse at the yolk stalk around stage 24-28.

Lumenal gut differentiation creates three morphologically and physiologically distinct regions: foregut, midgut and hindgut. The foregut and hindgut are derivatives of the primitive gut tubes initiated at the AIP and CIP, respectively. The midgut is derived from both foregut and hindgut primordia, formed from the union of the primitive gut tubes. Although most of the adult gut epithelium descends from definitive endoderm, the most anterior (part of the oral epithelium) and posterior (part of the cloacal epithelium) are ectodermally derived (Romanoff, 1960).

The hindgut develops into large intestine and forms part of the cloaca, the common gut-urogenital opening. The midgut/hindgut border is demarcated by the ceca, a paired tubal structure analogous to the mammalian appendix. These form as budding expansions at stage 19-20. Anterior to the ceca, the midgut develops into the small intestine. Pharynx,

esophagus, crop, gizzard and glandular stomach are foregut derived. These regions of chick gut can be distinguished by gross morphologic differences as early as day 3 of incubation (stage 23). Microscopic and physiologic distinctions, generally characterized by the epithelial phenotype of each region, do not become apparent until day 11 of incubation (Romanoff, 1960).

Normal gut tube formation and regionalization is dependent on interactions between the endoderm and mesoderm (Haffen et al., 1987). Although much of the evidence of this epithelial-mesenchymal interaction describes the dependence of epithelial phenotype on mesodermal signals (late patterning events), the initial events in gut morphogenesis, AIP and CIP induction, may involve endoderm signaling to mesoderm. For example, primitive endoderm signals adjacent mesenchymal tissues to undergo gut-specific mesodermal differentiation (Haffen et al., 1983; Kedinger et al., 1986, 1990). These early epithelial-mesenchymal inductive signals are unknown.

The protein product of the *Sonic hedgehog* gene, a homolog of the *Drosophila* segment polarity gene *hedgehog* (*hh*), is an excellent candidate for an early endodermally derived inductive signal in gut morphogenesis. Sonic is a signaling molecule implicated in mediating pattern in several regions of the embryo including the limb bud (Riddle et al., 1993), somite (Johnson et al., 1994; Fan and Tessier-Lavigne, 1994) and neural tube (Echelard et al., 1993; Kraus et al., 1994; Roelink et al., 1994). Sonic is an intriguing candidate for an inductive signal in the gut as it is expressed at the earliest stages of gut development, in the endoderm of the AIP and CIP (Riddle et al., 1993; Echelard et al., 1993).

Members of the *TGF-β* superfamily are key downstream targets of hedgehog genes in several species. In *Drosophila, hh* controls pattern in the imaginal discs in part by activation of the *TGF-β* relative *decapentaplegic* (*dpp*) (Basler and Struhl, 1994; Diaz-Benjumea et al., 1994; Heberlein et al., 1993; Ma et al., 1993; Tabata and Kornberg, 1994). *dpp* also has a critical patterning role in *Drosophila* midgut morphogenesis (Staehling-Hampton et al., 1994; Staehling-Hampton and Hoffmann, 1994; Tremml and Bienz, 1989; Immergluck et al., 1990; Panganiban et al., 1990). In vertebrates, *dpp* has two homologs, *Bmp-2* and *Bmp-4*. *Sonic* has been shown to induce ectopic expression of *Bmp-2* when misexpressed in the chick limb (Laufer et al., 1994). Since this inductive pathway has been so widely conserved, *Bmp-2* and *Bmp-4* may also be downstream targets of *Sonic* in the vertebrate gut.

The control of regionalization of the gut along the anterior-posterior (AP) axis is poorly understood. *Hox* genes have been implicated as key regulators of AP pattern in the limb bud, axial tissues and the hindbrain (reviewed in Krumlauf, 1994; McGinnis and Krumlauf, 1992). In the vertebrate limb, Sonic is thought to regulate AP pattern in part by activating *Hoxd* genes in the mesoderm (Riddle et al., 1993). A careful analysis of *Hoxa9-Hoxa13* expression has been carried out in the developing chick hindgut where the borders of their expression were found to match the morphologic boundaries of the gut (Yokouchi et al., 1995). Some of the genes in the *Hoxd* cluster have also been noted to be expressed in the murine hindgut (Dolle et al., 1991, 1993; Izpisua-Belmonte et al., 1991) although detailed temporal and regional expression patterns were not described. As *Hox* genes are known to have a role in patterning in other embryonic regions, their expression in the hindgut suggests that *Hox* genes might be involved in patterning the vertebrate hindgut, and may be activated by Sonic in the gut as they are in the limb bud.

We have investigated the roles of *Sonic*, *Bmp-2*, *Bmp-4* and the *Abd-B*-like *Hox* genes (*Hoxa-9*, *Hoxa-10*, *Hoxa-11*, *Hoxa-13*; *Hoxb-9*; *Hoxc-9*, *Hoxc-10*, *Hoxc-11*; *Hoxd-9*, *Hoxd-10*, *Hoxd-11*, *Hoxd-12*, *Hoxd-13*; *Hoxc-12* and *Hoxc-13* not studied) in chick hindgut formation by examining their expression patterns. We test potential interactions by altering their expression through virally mediated *Sonic* misexpression.

MATERIALS AND METHODS

Embryos

Chick embryos were obtained by incubation of fertilized White Leghorn eggs (Spafas) and were staged according to Hamburger and Hamilton (1951).

Probes

The *Sonic* clone has been previously described (Riddle et al., 1993). *BMP* probes were isolated using primers designed to amplify members of the *TGF-β* and *BMP* families (Basler et al., 1993). Eight independent 120 bp *BMP* fragments were amplified from a stage 22 chicken posterior limb bud plasmid cDNA library. These fragments were pooled and used to screen an unamplified stage 22 limb bud λZap cDNA library constructed as in Riddle et al. (1993). Among the *BMP*-related clones isolated were chicken *Bmp-2* (Laufer et al., 1994) and *Bmp-4* (Francis et al., 1994). Both clones contain the entire coding regions. Chick *Hox* genes (see list above) were isolated from stage 24 chick limb cDNA libraries (Nelson, C. et al., unpublished data). DNA templates used to generate RNA probes are described elsewhere (Burke et al., 1995). Digoxigenin-UTP-labeled RNA probes were transcribed as per Riddle et al. (1993).

Whole-mount in situ hybridization

Harvested chick embryos were harvested and fixed overnight in 4% paraformaldehyde in PBS, washed in PBS and processed for whole-mount in situ hybridization as previously described (Riddle et al., 1993). Embryos were photographed from either ventral or dorsal surfaces under transmitted light using a Nikon zoom stereomicroscope with Kodak Ektar 100 ASA film. Following whole-mount in situ hybridization, embryos and viscera were processed for sectioning as previously described (Riddle et al., 1993). 15-25 μm transverse sections were air dried and photographed with bright-field or Nomarski optics using a Zeiss Axiophot microscope and Kodak Ektar 25 ASA film.

CIP endoderm transplants

Stage 13 chick embryos were harvested into sterile Tyrodes solution, transferred into Tyrodes solution containing 0.3 mg/ml collagenase and incubated at 37°C for 30-60 minutes. Embryos were washed twice in Tyrodes solution containing 10% sheep serum, then placed in fresh serum-free Tyrodes solution for dissection. Using fine forceps, endoderm was dissected from mesoderm and ectoderm, from mid-embryo level caudally. The endoderm was trimmed to include only the most caudal area (the CIP). Representative CIP endodermal fragments were tested for purity by frozen section. The CIP endoderm fragments were stained in 1% Nile blue in Tyrodes solution for 30 minutes then transplanted into the anterior proximal region of the right wing bud of stage 20-24 chick embryos in ovo (Riddle et al., 1993). Transplanted embryos were incubated for 7 days, harvested into PBS, fixed in 4% paraformaldehyde, cleared and stained for cartilage as previously described (Riddle et al., 1993).

Embry cultur

Stage 8-13 embryos were harvested and cultured with their ventral surface facing up following the technique of New (1955). Cultures were incubated in a humidified chamber at 37°C after experimental manipulation (see below). Embryos were cultured as long as possible (generally 18-36 hours) before harvesting.

Retroviral misexpression studies

A retroviral vector engineered to express a full-length cDNA of chicken *Sonic* (Riddle et al., 1993) was injected into cultured stage 8-13 chicken embryos targeting the definitive endoderm at a mid-embryo level on the embryo's left. Approximately 0.1-0.2 μl of viral stock (titered at $1\text{-}2\times10^8$ cfu/ml) was injected per embryo. Embryos were cultured for 18-36 hours at 37°C (see above), harvested into PBS and processed for whole-mount in situ hybridization (Riddle et al., 1993).

RESULTS

Sonic and *Bmp-4* are expressed in adjacent tissues in the developing chick hindgut

The induction of the AIP and CIP is the earliest event in gut development. *Sonic* has been noted to be expressed in the endoderm of the early vertebrate gut in the region of the AIP and CIP (Riddle et al., 1993; Echelard et al., 1993). We used in situ hybridization to characterize further its temporal and spatial gut expression pattern. *Sonic* expression is first detected in the primitive endoderm in the anterior and caudal regions of the embryo. At stage 10, the AIP is formed and Sonic can be detected along its endodermal lips. The expression at the presumptive CIP could be detected at stage 10 in the caudal endoderm peripheral to the overlying nascent tailbud, before morphologic infolding is visible. *Sonic* continues to be expressed as the CIP forms at stage 13, where it becomes restricted to the endoderm of the fold (Fig. 1). *Sonic* expression is subsequently detectable throughout the gut endoderm in all stages examined and in adult chick gut epithelium (with highest levels detected in the crypts and base of intestinal villi, data not shown).

There is strong evidence that the hedgehog signaling pathway is conserved across species (for review see Ingham, 1995). We investigated the possibility that *Bmp-2* and *Bmp-4*, homologs of *dpp* in the *Drosophila hh* signaling pathway, may be involved in vertebrate gut development. *Bmp-2* is not expressed in the gut at the AIP or CIP (data not shown), whereas *Bmp-4* is expressed at these critical areas of gut formation. The earliest detectable expression of *Bmp-4* is simultaneous with the first observable expression of *Sonic*. In the developing hindgut, *Bmp-4* expression is restricted to the mesoderm abutting the endoderm expressing *Sonic* at the CIP and is expressed in a domain slightly more anterior and lateral than that of *Sonic*. The expression of *Bmp-4* becomes restricted to the mesoderm in and immediately adjacent to the CIP as the CIP becomes morphologically distinct (stage 13, Fig. 1B). As the CIP elongates anteriorly to form a lumen, *Bmp-4* expression also expands anteriorly, remaining restricted to the gut mesoderm. The tissue restricted expression of both *Sonic* in the endoderm and *Bmp-4* in the mesoderm is maintained into mid-developmental stages (stages 28-33) and is present throughout the lumenal gut from the pharynx to the cloaca. *Bmp-4* expression is still detectable at stages 28-33 but the signal is weak. We were unable to detect *Bmp-4* expression in adult chick gut tissues examined (data not shown).

Sonic expr ssion is sufficient to induc *Bmp-4* expression in the presumptive visceral mesoderm

The expression of *Sonic* and *Bmp-4* in adjacent tissues is consistent with the possibility that Sonic induces *Bmp-4* expression in the gut. We used virally mediated misexpression to test whether Sonic is capable of inducing *Bmp-4* in the visceral mesoderm. A replication competent retrovirus engineered to express *Sonic* (Riddle et al., 1993) was injected at mid-embryo near the insertion of the left vitelline vein in stage 8-13 chick embryos cultured in vitro (New, 1955). The injection site targeted a region that does not express either *Sonic* or *Bmp-4* at these stages. Embryos were harvested 18-36 hours later and the expression patterns of *Sonic* and *Bmp-4* were examined by whole-mount in situ hybridization. Endogenous endodermal *Sonic* expression could be detected at the AIP and CIP, and ectopically at the site of viral injection in both the endoderm and mesoderm (Fig. 2A). *Bmp-4* expression is seen induced specifically in the mesoderm at the site of injection, in addition to its normal expression in the mesoderm of the CIP (Fig. 2B).

The *Abdominal-B* class of *Hox* genes are regionally restricted in their expression in the hindgut

As the gut forms, it develops into morphologically and functionally distinct regions. Regionalization of many other embryonic tissues is regulated by *Hox* gene expression (Krumlauf, 1994; McGinnis and Krumlauf, 1992). To evaluate the possible roles of the *Hox* genes in the development and regionalization of the hindgut, and to examine their potential relationship to *Sonic* expression in the hindgut endoderm, we examined the expression of the *Abd-B*-related *Hox* genes during chick hindgut development.

The *Abd-B*-related *Hox* genes expressed in the hindgut are all initially expressed in the caudalmost mesoderm of the embryo around the nascent CIP. None are expressed prior to the initiation of *Sonic* transcription in the CIP. Between stages 10 and 13, they are sequentially activated in a temporal order, collinear with their order on the chromosome. *Hoxd-9* is expressed before *Hoxd-10*, which is expressed before *Hoxd-11, Hoxd-12* and, finally, *Hoxd-13* (data not shown). Each gene is expressed in a posterior domain, in an overlapping, nested pattern around the CIP by stage 13. These expression domains encompass the caudal mesoderm destined to form the visceral mesoderm of the posterior gut, as well as probably contributing to other mesodermal structures. The anterior boundaries of expression matches the chromosomal relationship of the genes: *Hoxd-9* (most 3′ on the chromosome) with the most anterior expression boundary, and *Hoxd-13* (most 5′) the most posteriorly restricted (Fig. 3). At stage 13, there is no morphologic distinctions within the primitive hindgut. Morphologically distinct regions develop and are discernible by stage 23.

The relative anterior boundaries of expression, present by stage 13, are maintained during gut morphogenesis. At stage 25, *Abd-B*-like genes of the *Hoxa* and *Hoxd* cluster (Fig. 4A) are regionally restricted in their expression in hindgut mesoderm with sharp expression boundaries at the borders of morphologically distinct portions of the hindgut (Fig. 4B,C). The most anteriorly expressed gene, *Hoxa-9*, has an anterior border of expression within the mesoderm of the posterior

midgut (at a point approximating the distal third of the midgut length). Each successive gene within the *Hoxa* and *Hoxd* clusters has a more posterior boundary of expression. *Hoxa-10*, *Hoxd-9* and *Hoxd-10* are restricted in their expression to the ceca. *Hoxa-11* and *Hoxd-11* have an anterior limit of expression in the mid-ceca at the approximate midgut/hindgut boundary (Romanoff, 1960). *Hoxd-12* has an anterior limit at the posterior border of the ceca and extends posteriorly throughout the hindgut to the cloaca. *Hoxa-13* and *Hoxd-13* are expressed in the most posteriorly restricted domain, in ventral mesoderm surrounding the cloaca. *Hoxa-13* and *Hoxd-13* are the only *Abd-B*-like genes that are also expressed within the gut endoderm, from the cloaca to the ceca. The expression patterns of *Hoxc-12* and *Hoxc-13* were not examined. The domains of expression of the *Hoxa* genes are essentially the same as those described by Yokouchi et al. (1995). This expression of *Hoxd-13* is similar to that reported in the mouse (Dolle et al., 1991, 1993).

The only *Abd-B*-like member of the *Hoxb* and *Hoxc* clusters that we found to be expressed in the hindgut is *Hoxc-9* (see

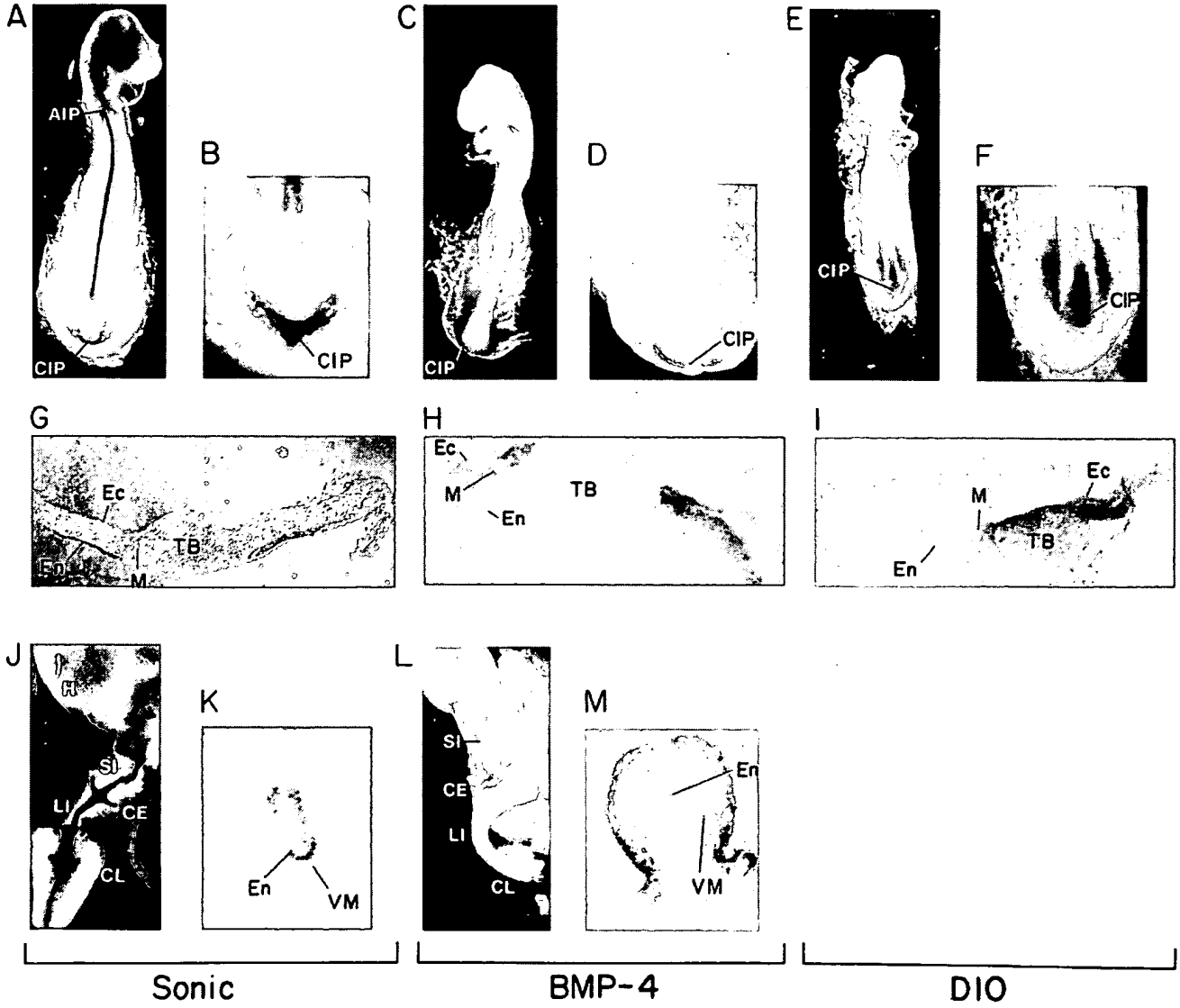


Fig. 1. Expression of *Sonic*, *Bmp-4* and *Hoxd-10* in stage 13 chick embryos determined by whole-mount in situ hybridization. *Sonic* expression is detected in the endoderm of the AIP and CIP in pre-gut closure stage embryos seen at low (A) and high magnification (B), ventral view. Endodermal expression confirmed in section (G). At later stages *Sonic* is expressed in the gut in all levels (foregut, midgut and hind-gut) as shown in a stage 28 embryo (J) restricted to the endoderm, confirmed in section (K). *Bmp-4* is expressed in the mesoderm adjacent to *Sonic* at the CIP in the ventral mesoderm seen at low (C) and high magnification (D), dorsal view. Mesenchymal expression is confirmed in section (H). *Bmp-4* gut expression persists but weakens in later stage embryos as shown in a stage 33 embryo (L), in the visceral mesoderm only (M). *Hoxd-10* is expressed in the caudal end of the embryo in the tailbud and peripheral mesoderm around the CIP (E,F) confirmed in section (I). In later stages, the expression of *Hoxd-10* is restricted to the ceca, see Fig. 4. AIP, anterior intestinal portal; CE, ceca; CIP, caudal intestinal portal; CL, cloaca; Ec, ectoderm; En, endoderm; H, heart; LI, large intestine; M, mesoderm; SI, small intestine; TB, tailbud; VM, visceral mesoderm.

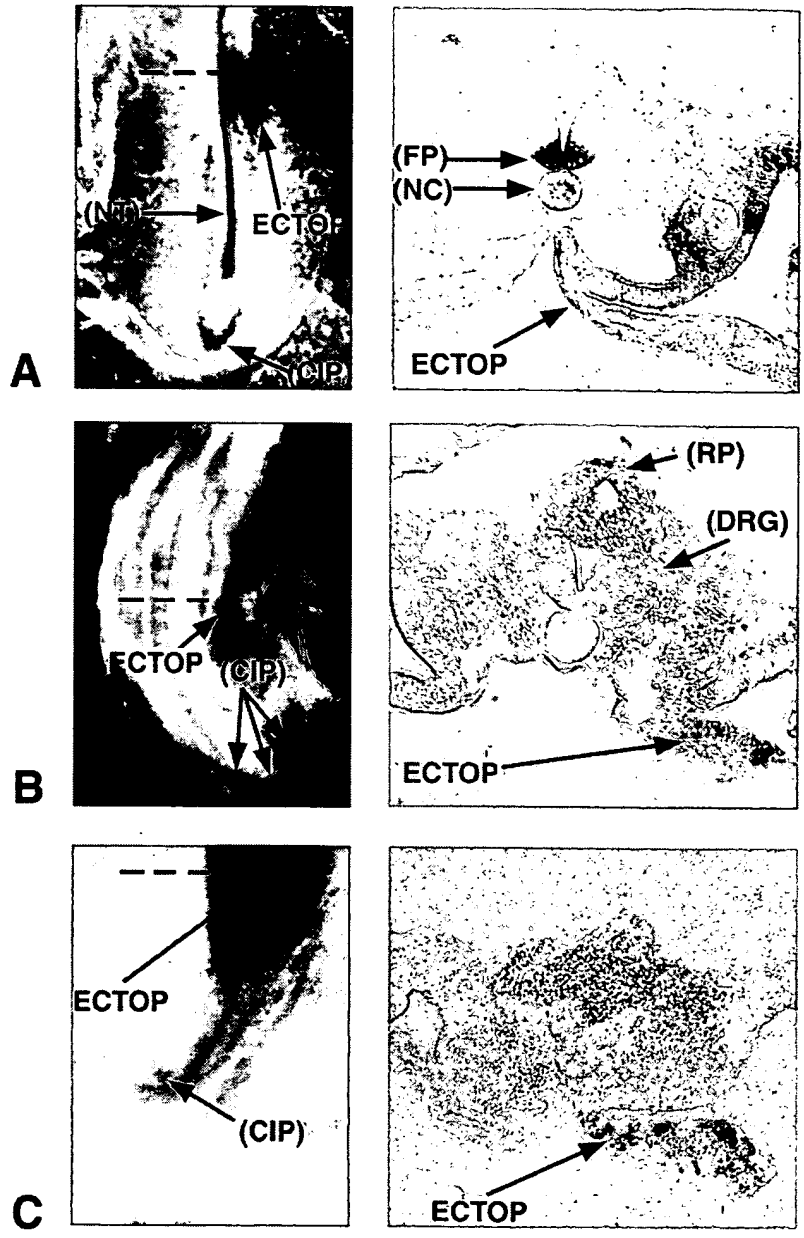


Fig. 2. Misexpression of *Sonic* induces ectopic expression of *Bmp-4* and *Hoxd-13* in mesodermal tissues of the developing chick. (A) Chick embryo cultured at stage 10, injected with *Sonic* virus at mid-embryo level on left ventral surface, harvested after 24 hours in culture and processed for whole-mount in situ hybridization with a *Sonic* probe. The normal endogenous expression of *Sonic* is detected at the AIP, CIP and in the midline (neural tube and notochord, see right panel). Ectopic *Sonic* expression is present unilaterally on the left ventral surface. 25 μm section of a similarly injected embryo. Endogenous *Sonic* expression is seen in the floor plate of the neural tube and notochord. Ectopic expression is seen unilaterally in the visceral endoderm, its underlying splanchnic mesoderm and somatic mesoderm. (B) Chick embryo infected with *Sonic* virus and hybridized with a *Bmp-4* probe. Normal endogenous expression is seen in the mesoderm of the CIP, and ectopically at the site of *Sonic* virus injection. Section through a similar embryo showing normal endogenous *Bmp-4* expression in the roof plate of the neural tube and the forming dorsal root ganglia. Induced *Bmp-4* expression is present unilaterally in the splanchnic mesoderm at the site of *Sonic* viral injection, and not in the visceral endoderm. (C) Chick embryo injected with *Sonic* virus and hybridized with a *Hoxd-13* probe. Ectopic *Hoxd-13* is induced unilaterally in addition to the endogenous CIP signal. Section through a similar embryo showing induced *Hoxd-13* expression in the visceral mesoderm (shown) and focally in the gut endoderm (data not shown). Labels in parenthesis indicate normal endogenous expression pattern. Those without parenthesis indicate ectopic/induced expression. Dashed lines indicate approximate plane of section. CIP, caudal intestinal portal; DRG, forming dorsal root gangia; Ecto, ectopic expression; FP, floor plate of the neural tube; NC, notocord; RP, roof plate of the neural tube.

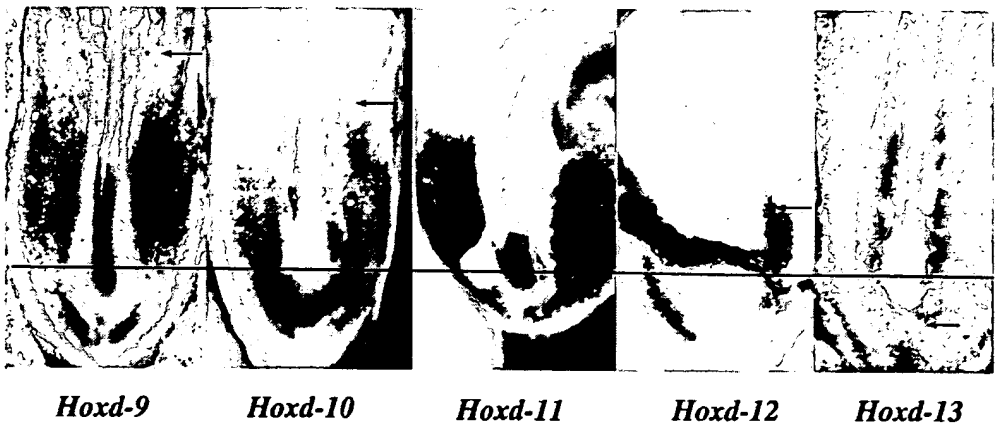


Fig. 3. Nested expression of *Hoxd* genes at the CIP. Stage 12 chick embryos were harvested and hybridized with probes for *Hoxd-9, Hoxd-10, Hoxd-11, Hoxd-12 and Hoxd-13*. Alligned by tailbud (thin line), the anterior expression boundaries are highlighted by arrows. The expression limits are nested around the CIP-expressing *Sonic* (see Fig. 1).

Fig. 4B). The expression of *Hoxc-9* overlaps with its paralogues *Hoxa-9* and *Hoxd-9* in the midgut mesoderm, but has a sharp posterior boundary in the mid-ceca, complementary to *Hoxa-11* and *Hoxd-11* (Fig. 4B).

Sonic expression is sufficient to induce *Hox* expression in the gut

Sonic is expressed at the CIP (in the endoderm), around which the *Hox* genes are expressed in a nested pattern in the pre-gut mesoderm. This early expression pattern is reminiscent of the nested expression of the *Hoxd* genes centered around the posterior of the limb bud (Dolle et al., 1991; Izpisua-Belmonte et al., 1991a; Nohno et al., 1991). The posterior limb bud produces Sonic hedgehog, which is sufficient to trigger the expression pattern of these genes (Riddle et al., 1993). This suggests that Sonic may also initiate the expression of these *Hox* genes in the hindgut.

To test whether Sonic is capable of inducing *Hox* expression in the gut mesoderm, *Sonic*-expressing virus was injected unilaterally through the presumptive endoderm into the mesoderm at a mid-embryo level of stage 8-13 chick embryos cultured in vitro (New, 1955). At these stages, the region targeted for misexpression does not yet express *Sonic*, *Bmp-4*, *Hoxd-11* or *Hoxd-13*. When embryos were examined by in situ hybridization, ectopic *Hoxd-11* (data not shown) and *Hoxd-13* (Fig. 2C) expression could be detected within the visceral mesoderm at the site of injection, in addition to their normal regional expression within the gut mesoderm. This induction appears to be sensitive to the AP level of the injection site as ectopic expression of *Hoxd-11* or *Hoxd-13* was not detected in a limited number of injections of *Sonic* virus anterior to the vitelline veins (data not shown).

Th gut endoderm can act as a polarizing center

Sonic can induce ectopic *Bmp-4*, *Hoxd-11* and *Hoxd-13* expression in the mesoderm after infection of both endodermal and mesodermal tissues (Fig. 2B). As the endogenous expression of *Sonic hedgehog* is restricted to the endoderm, these experiments do not address whether the endoderm alone is competent to act as a source of a functional Sonic protein. It has been previously shown that cells producing active Sonic protein can induce duplications when transplanted into the anterior margin of early limb buds (Riddle et al., 1993). A variety of tissues that express *Sonic*, such as the floor plate of the neural tube, the notochord and Hensen's node, can induce such duplications (Hornbruch and Wolpert, 1986; Saunders and Gasseling, 1983; Stocker and Carlson, 1990; Wagner et al., 1990).

To test whether gut endoderm, which expresses *Sonic* message, also produces a polarizing signal consistent with functional Sonic protein (Lee et al., 1994), CIP endoderm was manually dissected from the embryo. When this isolated CIP endoderm is transplanted into the anterior of a host stage 20-24 chick limb bud, mirror-image digit duplications are induced (Fig. 5), suggesting that the CIP is a source of active Sonic protein.

DISCUSSION

Gut morphogenesis is dependent on inductive epithelial-mesenchymal interactions. We have shown that Sonic hedgehog, a known signaling molecule, is an excellent candidate for a molecule mediating critical aspects of gut development. *Sonic* is expressed in the definitive endoderm in the early embryonic areas where gut formation begins, at the anterior and posterior ends of the embryo. This expression pattern becomes restricted to the endoderm of the first identifiable gut regions, the AIP and CIP, and remains restricted to the endoderm as the gut tube forms.

Sonic induces mesodermal *Bmp-4* expression in th hindgut

We provide evidence that a target of Sonic, produced by the gut endoderm, is an inductive signal acting on the adjacent visceral mesoderm. One downstream target of Sonic is *Bmp-4*. *Bmp-4* is expressed in the visceral mesoderm early in its formation. The expression domains of *Sonic* and *Bmp-4* abut across tissue layers just before the earliest morphologically identifiable formation of the CIP. Subsequently, *Bmp-4* is expressed throughout the gut mesoderm during gut morphogenesis. In addition to their normal simultaneous expression in adjacent gut tissues, we show that misexpressed *Sonic* has the ability to induce ectopic expression of *Bmp-4* in the visceral mesoderm. Together these facts strongly suggest that endodermally derived Sonic protein normally functions to induce mesodermal *Bmp-4* expression during formation of the gut tube. Bmp-4 is itself a secreted protein, implying it may be a secondary signal in an inductive cascade. Bmp-4 could act either as part of a feedback loop to the endoderm or within the visceral mesoderm. This latter possibility is consistent with the finding that the ventral mesoderm fails to close in mice homozygous for a deletion in the *Bmp-4* gene (Hogan, Blessing, Winnier and Labosky, personal communication). *Sonic* may thus serve as a signal from the endoderm to recruit visceral mesoderm by inducing expression of *Bmp-4*, which in turn initiates growth or specification of the visceral mesoderm. This may provide a molecular explanation for the experimental findings that the primitive gut endoderm is capable of signaling underlying mesoderm to induce visceral-specific mesodermal differentiation (Haffen et al., 1983; Kedinger et al., 1986, 1990).

The induction of *Bmp-4* by Sonic hedgehog in the lumenal gut is one of a growing number of examples of members of the *BMP* family as downstream targets of *hedgehog* gene products. Elsewhere in the vertebrate embryo, it is the closely related gene *Bmp-2* that is a downstream target of *Sonic*. For example, *Bmp-2* is expressed in response to *Sonic hedgehog* in the vertebrate limb bud (Laufer et al., 1994). In *Drosophila*, the homolog *dpp* is activated by *hh* in the imaginal discs (Basler et al., 1994; Diaz-Benjumea et al., 1994; Heberlein et al., 1993; Ma et al., 1993; Tabata and Kornberg, 1994). The use of this same pathway among phylogenetically divergent organisms suggests this cascade of signaling molecules has been evolutionarily conserved and co-opted for various purposes in the regulation of developmental processes.

Sonic induces *Hox* gene expression in the hindgut

Other genetic targets of *Sonic* include members of the *Abd-B* class of *Hox* genes, which we show here to be expressed in mesodermal tissues of the gut. Early in gut formation (stages 10-14), when the *Hox* gene expression is first detected, the expression of *Sonic* is limited to the posterior of the presumptive hindgut at the CIP. At these early time points, the *Hox* genes are expressed in a nested pattern around the CIP-

Sonic signal, in a spatial organization reminiscent of their spatial relationship to the ZPA in the limb. This suggests that, as in the limb bud, *Sonic* may act to induce *Hox* gene expression in the hindgut mesoderm. Consistent with this model, we find that misexpression of *Sonic* is sufficient to cause the ectopic expression of *Abd-B* like *Hox* genes in the early gut mesoderm.

Subsequently, *Sonic* is expressed uniformly throughout the gut endoderm along the AP axis while the *Hox* genes retain a restricted expression pattern. The fact that the transcriptional domains of *Hox* genes are only nested around cells expressing *Sonic* early in gut development suggests that the mesoderm may have a limited time window during which it is competent to respond. There must also be a spatial restriction to this competence, as these genes are not activated around the AIP, which also expresses *Sonic* in the endoderm. Our preliminary results support this as anteriorly injected *Sonic*-expressing virus fails to activate ectopically *Hoxd-13* yet is able to activate *Bmp-4* (data not shown). Another example of regional restriction of response to *Sonic hedgehog* is seen in the embryonic midline. *Sonic* is expressed throughout the developing notocord, yet *Abd-b*-like *Hox* genes are not activated in the paraxial mesoderm as they are in the limb mesenchyme and in the visceral mesoderm abutting the CIP.

Hox gene expression domains in the hindgut demarcate morphologic boundaries

Once the nested expression domains of the *Abd-B*-related *Hox* genes are established, their relative anterior borders of expression are maintained through subsequent growth and differentiation. It should be noted, in this regard, that the entire embryo undergoes enormous growth during the stages studied and additionally there is considerable growth of the tailbud caudally. Hence the absolute distance between the boundaries of the *Hox* genes, initiated around the CIP, increase during gut morphogenesis (Fig. 6). The expression patterns strongly suggest that these genes may have a role in gut patterning. Once the gut tube is formed, it becomes regionalized along the anteroposterior axis. These regions are distinct in their gross and microscopic morphology and function, and include differences in both mesodermal and endodermal differentiation.

The restricted boundaries of expression of the *Abd-B*-like *Hox* genes in the gut appear to demarcate the regions that will form the cloaca, large intestine, ceca, mid-ceca at the midgut/hindgut border and the lower portion of the midgut (perhaps identifying that portion of the midgut derived from the posterior gut tube; Romanoff, 1960). Moreover, these molecular events presage regional distinctions. Expression of all *Hox* genes could be detected by stage 14, well before the hindgut lumen is closed (by stage 28). Cytodifferentiation of the hindgut mesoderm and epithelium begins later, at stages 29-31 (Romanoff, 1960).

The expression patterns of *Hox* genes in the hindgut are dynamic. For example, the expression domains of *Hoxd-10* and *Hoxa-10,* which originally extend to the posterior limit of the gut, resolve into restricted domains solely encompassing the ceca. The refinement of the *Hox* expression patterns may involve cross-regulation between the *Hox* genes, or may be due to secondary factors.

While the regionalized expression of most of the *Abd-B* class of *Hox* genes in the midgut and hindgut is limited to the mesoderm, their expression may influence regional specification of the endoderm as well. Vertebrate gut mesoderm has been demonstrated to provide cues that influence the regional differentiation of the endoderm. For example, intestinal mesenchyme induces heterodifferentiation of primitive endoderm in co-culture experiments (Haffen et al., 1982, 1983; Ishizuya-Oka and Mizuno, 1984; Kedinger et al., 1986; Takiguchi et al., 1988; Takiguchi-Hayashi et al., 1990; Yasugi, 1984).

Our results suggest that specific *Hox* genes are activated in temporal sequence by *Sonic*. The restricted *Hox* expression domains around morphologic borders of the gut suggest that they may be responsible for regulating morphogenesis of the gut. Consistent with this, there is an apparent homeotic alteration in the gut of a transgenic mouse in which the anterior limit of expression of *Hoxc-8* is shifted rostrally: a portion of foregut epithelium mis-differentiates as midgut (Pollock et al., 1992). In the chick *Hoxc-8* is normally expressed in the midgut, with a sharp anterior expression boundary at the duodenum (data not shown).

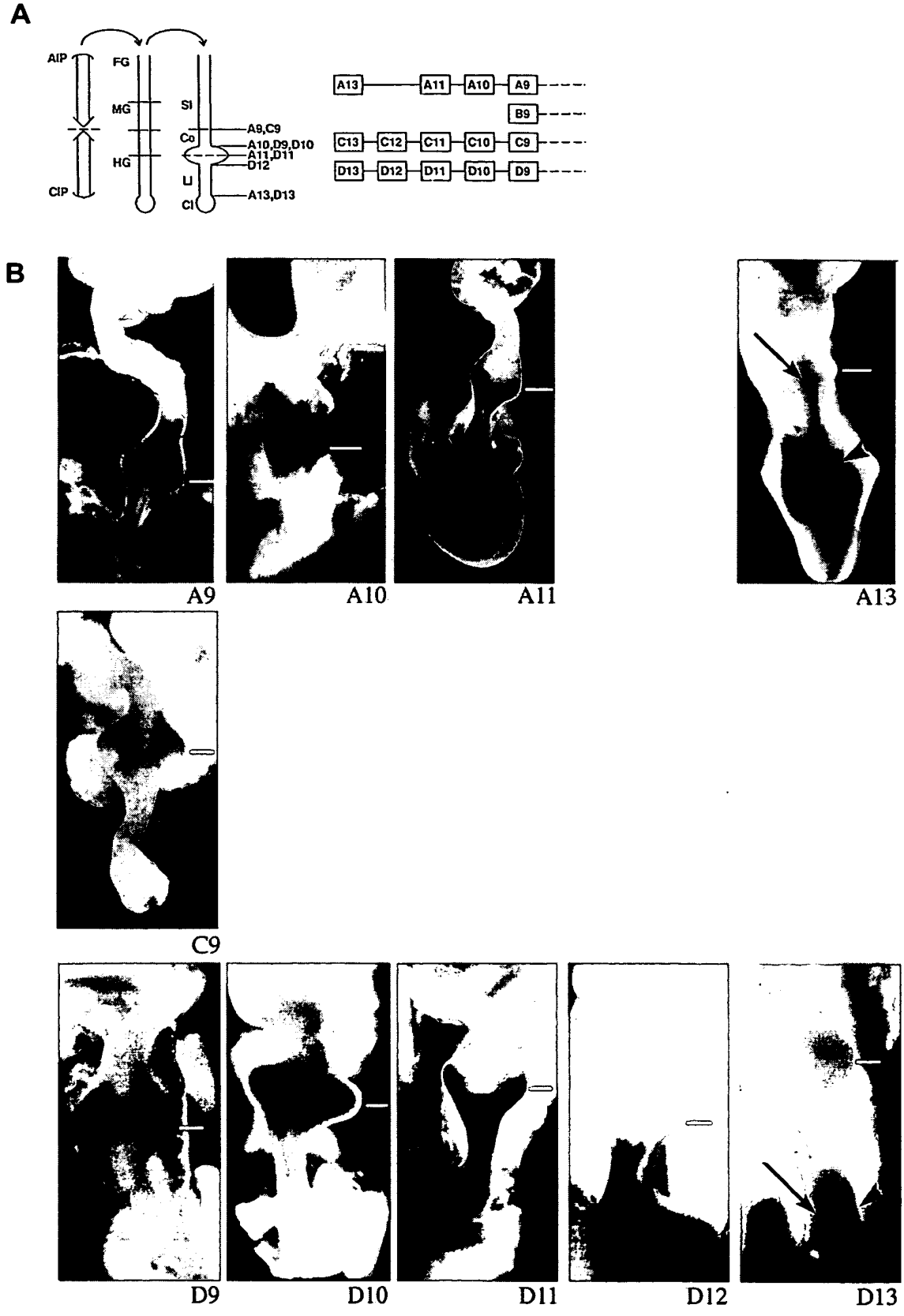
Abd-b-like *Hox* genes expression is also initiated adjacent to the CIP in lateral mesodermal tissue. Our study did not investigate the expression of these *Hox* genes in other visceral tissues nor did it explore the correlations between those expression domains and morphologic structures. However, based on their initial caudal expression, it is possible that Sonic protein produced at the CIP plays a role in establishing other (non-gut) caudal visceral mesodermal *Hox* gene expression domains as well.

Initiation and transduction of *Sonic* signal in the gut

Previous studies have shown that the transcription factor HNF-3β is also expressed in, and may be responsible for, the induction of early gut endoderm (Ang et al., 1993). In the mouse, HNF-3β is expressed in the AIP and CIP (Ang et al., 1993; Monaghan et al., 1993; Sasaki and Hogan, 1994). It has been suggested that HNF-3β regulates the production of Sonic by the notochord and floorplate (Echelard et al., 1993; Kraus et al., 1993; Monaghan et al., 1993); HNF-3β expression may similarly lead to transcription of Sonic within the gut endoderm.

Once secreted, Sonic protein serves as a signal to adjacent cells. A candidate receptor for *hh* in *Drosophila* is *patched* (*ptc*) (Taylor et al., 1993). The vertebrate homolog of *ptc* has recently been cloned and its expression pattern described (Goodrich, Johnson, Milenkovic and Scott, unpublished data; Marigo, Scott, Johnson, Goodrich and Tabin, unpublished data). *Ptc* is expressed in the visceral mesoderm immediately subjacent to *Sonic*'s endodermal expression in the developing chick and mouse hindgut. This further supports the supposition that Sonic acts as an epithelial-mesenchymal signal in gut development.

BMP-4 is a secreted factor produced in the visceral mesoderm in response to endodermally derived Sonic protein. BMP-4 could, therefore, be an intermediary signal in the pathway inducing *Hox* gene expression. If BMP-4 does act in organizing the *Hox* gene expression, it is likely to do so early when its mRNA expression is posteriorly localized. Its subsequent expression pattern would be consistent with a role in maintenance of established *Hox* expression patterns. Interestingly, the highly related gene *Bmp-2* is expressed in the posterior of the limb bud in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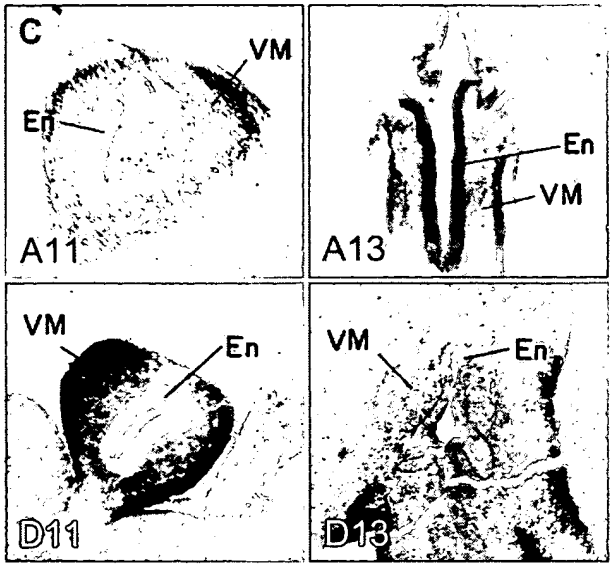

Fig. 4. Expression of *Hox* genes in the developing chick gut. (A) Diagrammatic representation of vertebrate gut morphogenesis with the anterior intestinal portal and caudal intestinal portal growing and elongating (large arrows) towards the umbilicus (dashed horizontal line). The regionalization of the lumenal gut forms foregut, midgut and hindgut derived from the invaginations of the AIP (foregut and midgut) and CIP (midgut and hindgut). Cytodifferentiation of these regions forms the small intestine from the midgut, the ceca from both midgut (anteriorly) and hindgut (posteriorly), and large intestine and part of the cloaca from hindgut. The regionally restricted pattern of expression of the *Abd-B* like *Hox* genes demarcates morphologic distinctions in the midgut and hindgut visceral mesoderm, diagrammatically shown with anterior limits of expression noted (exception is *Hoxc-9*, posterior limit of expression shown). The genes expressed in the vertebrate abdominal region are from the 5′ end of each cluster, related evolutionarily to the *Drosophila* gene *Abd-B*. The cognate genes (paralogues) of the *Abd-B* like genes of the four *Hox* complexes are diagramed with the most posteriorly expressed (5′) genes aligned on the left. (B) Expression of the 5′ members of the *Hox* genes in stage 26-28 chick hindgut is studied by whole-mount in situ hybridization. Paralogues are aligned. There is no paralogue of *Hoxd-12* in the *Hoxa* cluster. Paralogues without detectable hindgut expression (*Hoxb-9, Hoxc-10, Hoxc-11*) are not shown. *Hoxc-12* and *Hoxc-13* were not studied. Expression limits in the visceral mesoderm can be seen around the midgut and hindgut boundary of the ceca and the posterior limit of the hindgut, the cloaca. The ceca in each panel are highlighted by the thin white lines. In general, paralogues of different clusters exhibit similar anterior expression borders in the hindgut as they do in other embryonic tissues. *Hoxd-9* in the gut is an exception to this rule, as it is expressed in a domain seemingly identical to *Hoxa-10* and *Hoxd-10* at this stage. The functional significance of this deviation from the paralogue expression 'rule' is unclear. Expression of *Hoxa-13* and *Hoxd-13* is also found in the endoderm (long arrow indicates endodermal expression, arrowhead notes mesodermal expression. The anterior extent of *Hoxd-13* endodermal expression is not evident in this photograph). (C) Identification of the tissue layers expressing *Hox* genes by sectioned whole-mount in situ hybridization. On left *Hoxa-11* and *Hoxd-11* from stage 26-28 embryos expression is detected in the visceral mesoderm of the hindgut. The hindgut epithelium is not stained. On right, *Hoxa-13* and *Hoxd-13* from stage 26-28 embryos shows expression in the cloaca with both endoderm and ventral mesoderm staining. AIP, anterior intestinal portal; Ce, ceca; CIP, caudal intestinal portal; Cl, cloaca; EN, endoderm; FG, foregut; MG, midgut; HG, hindgut; LI, large intestine; SI, small intestine; VM, visceral mesoderm.

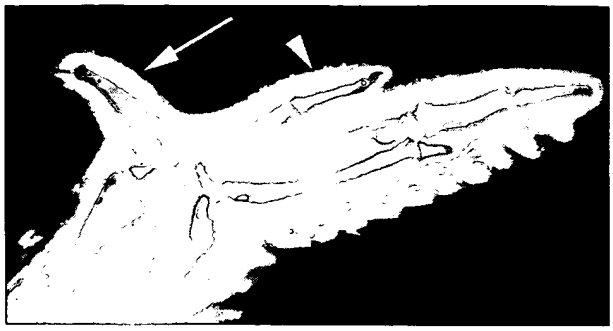

Fig. 5. The CIP produces active Sonic protein. The CIP endoderm was dissected from a stage 13 embryo and implanted into a host stage 24 chick embryo in ovo in the right anterior proximal limb bud, just under the anterior ectodermal ridge. The windowed egg was taped and incubated for 7 days at 37°C in a humidified chamber, harvested, fixed, cleared and stained for skeletal elements. A variety of phenotypes consistent with mirror-image duplications resulted as exemplified in this wing with a mirror-image duplicated digit two (arrow). Arrowhead, normal (wild type) digit two.

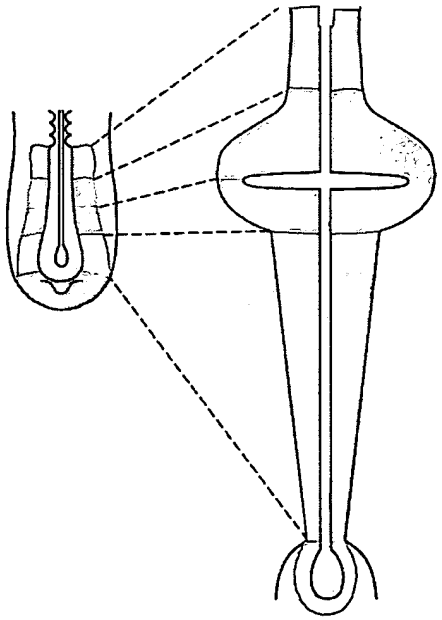

Fig. 6. Diagrammatic representation of the anterior limits of mesodermal expression of the *Abd-b*-like *Hox* genes studied herein at an early stage (approximately stage 14, lefthand figure) and a later stage (approximately stage 24, righthand figure). Colors respresent expression of paralogs (overlapping expression not represented). Red, paralog 13; Blue, paralog 12; Yellow, paralog 11; Green, paralog 10; Purple, paralog 9; (exceptions noted in text). The lines connecting the levels of expression between the two stages demonstrate that although expression boundaries expand with growth of the embryo, their relative morphologic boundaries are maintained.

response to *Sonic* (Laufer et al., 1994) and could play an analogous role in *Hoxd* induction or maintenance there. Mice carrying a homozygous deletion of the *Bmp-4* gene do not develop enough to assess whether there are defects in gut pattern in addition to defects in gut mesodermal closure (B. L. M. Hogan, M. Blessing, A. R. Winnier and P. A. Labosky, personal communication).

Signaling in insect and vertebrate gut development

There are intriguing parallels between the expression patterns of *Sonic, Bmp-4 and Hox* genes in the vertebrate gut and those of their homologs, *hh*, *dpp* and the *homeotic* genes, during *Drosophila* gut morphogenesis (Fig. 7). *hh* (like its vertebrate homolog *Sonic*) is expressed at the earliest stages of foregut and hindgut invaginations in the gut epithelium and may be a signal to visceral mesoderm (M. Scott, personal communication; P. Ingham, personal communication; T. Tabata and T. Kornberg, personal communication). *hh* is also expressed anterior and lateral to the developing anterior midgut (Mohler and Vani, 1992) and is required for *dpp* expression in the mesoderm of the gastric caecae (Pankratz and Hoch, 1995). Nothing is known directly of the relationship between *hh* expression and activation of expression of other genes in *Drosophila* foregut and hindgut. In the embryonic foregut of *Drosophila*, *dpp* is not expressed in the mesoderm but *hh* and *dpp* are co-expressed in the epithelium (Pankratz and Hoch, 1995). Although *hh* does not appear to induce *dpp* in the foregut, its role in the hindgut it not well characterized. *hh* could act to induce the expression of another member of the *BMP/dpp* family. One possible candidate is *60A*, a related gene which is expressed in the *Drosophila* foregut and hindgut mesoderm (Doctor et al., 1992; Wharton et al., 1991).

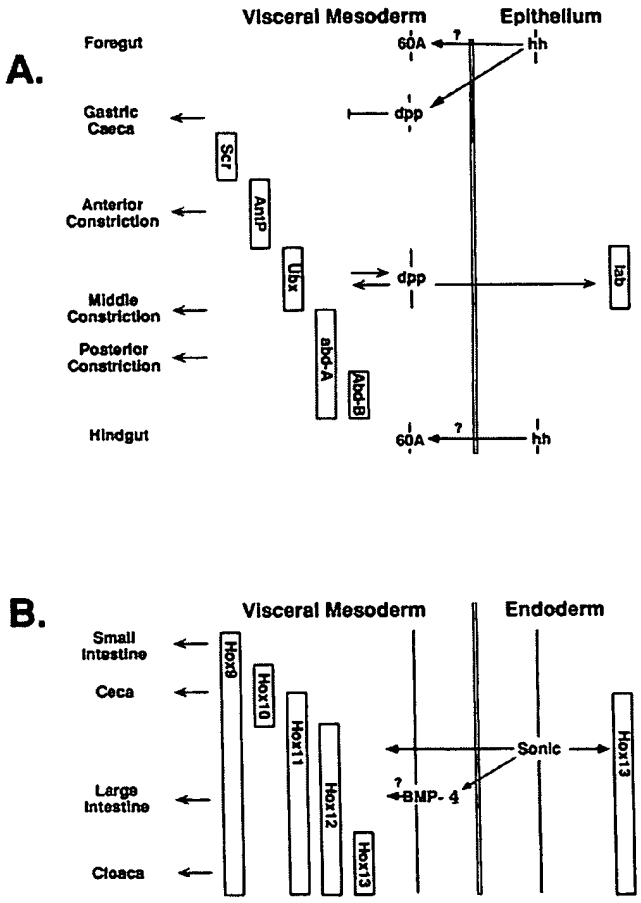

Fig. 7. Patterning of the *Drosophila* and vertebrate gut. Regulatory interactions responsible for patterning of *Drosophila* midgut (A) are compared to a model for patterning of the vertebrate hindgut (B). Morphologic regional distinctions are indicated to the left (A and B), genes expressed in the visceral mesoderm are in the center panel, those in the epithelium or endoderm are on the right. *HOM/Hox* gene expression domains are boxed. Regionally expressing secreted gene products are indicated by lines. Arrows indicate activating interactions, barred lines, inhibiting interactions. Regulatory interactions in *Drosophila* gut (A) have been established by genetic studies except for the relationship between *60A* and *hedgehog*. Hedgehog appears to be a signal from the epithelium to the mesoderm and *60A* is expressed in the mesoderm. The induction of *Hox* genes by Sonic may be mediated through an initial activation of *Bmp-4*.

Like its vertebrate homolog *Bmp-4*, *dpp* is expressed in the visceral mesoderm of the developing midgut. Later in *Drosophila* gut development, the production of dpp in the midgut mesoderm contributes to the regulation of the expression of homeotic genes in both the mesoderm and the endoderm (Immergluck et al., 1990; Panganiban et al., 1990; Staehling-Hampton et al., 1994; Staehling-Hampton and Hoffmann, 1994; Tremml and Bienz, 1989). Like the *Hox* genes in the vertebrate gut, borders of *Drosophila* homeotic gene expression correlate with gut morphologic boundaries. Although in *Drosophila* the homeotic gene expression domains are discrete, in the chick hindgut the expression domains of the *Abd-B*-like *Hox* genes are overlapping. The restricted homeotic gene expression in *Drosophila* midgut is known to determine the morphologic borders of the midgut (Bienz, 1994). Given the remarkable correlation with morphologic borders, it is likely that *Hox* gene expression also regulates aspects of vertebrate hindgut gut morphology.

The similarities in expression of *Sonic, Bmp-4 and Hox* genes to those of their *Drosophila* homologs suggests that the ancient common ancestor of *Drosophila* and vertebrates made use of those sets of genes in regulating gut morphogenesis. Some of their roles in this arena, such as the specification of region-specific morphogenesis by Hox/homeotic genes may have been conserved; while others, such as the expression of *BMP/dpp* may have been coopted for different regulatory functions. To the extent that the homologous genes play similar roles in the two organisms, pathways established by genetic studies in *Drosophila* will provide insights into the molecular basis for the regionalization and morphogenesis of the vertebrate gut.

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